Processes Underlying the Upregulation of Tom Proteins in *S. cerevisiae* Mitochondria Depleted of the VDAC Channel

Hanna Kmita,[1,2] Nina Antos,[1] Malgorzata Wojtkowska,[1] and Lilla Hryniewiecka[1]

It has been shown recently that in *Saccharomyces cerevisiae* mitochondria depleted of the VDAC channel (Δ*por1* mitochondria), the TOM complex channel substitutes for the VDAC channel. The additional function of the TOM complex channel is probably facilitated by the upregulation of nuclear-encoded components of the TOM complex as has been shown for Tom40 (a major component of the channel) and Tom70 (one of the surface receptors). Here we report that in *S. cerevisiae* cells the VDAC channel seems to be an important signal in the expression of the TOM complex components. *S. cerevisiae* cells depleted of the VDAC channel (Δ*por1* cells) contain distinctly increased levels of Tom40mRNA, and Tom70mRNA, but their synthesis and translation are affected differentially by the applied inhibitors of transcription and translation. Consequently, it may be concluded that depletion of the VDAC channel might influence differentially the expression of *TOM40* and *TOM70* genes.

KEY WORDS: TOM complex; VDAC channel; upregulation of Tom proteins; transcription, translation.

INTRODUCTION

Trafficking of molecules across the outer mitochondrial membrane is believed to be mediated primarily by channels. They participate not only in metabolite exchange between mitochondria and cytoplasm but also in protein import into these organelles. In spite of the functional complexity, the diversity of the outer membrane channels seems to be quite limited. The TOM complex channel (the channel of the translocase of the outer membrane) supports protein import (Paschen and Neupert, 2001; Pfanner and Chacinska, 2002; Rapaport, 2002), while metabolite transport is mediated by the VDAC channel (voltage dependent anion channel), known also as mitochondrial porin (Benz, 1994; Colombini *et al.*, 1996; Blachly-Dyson and Forte, 2001). The latter, however, may be present as isoforms encoded by separated genes and displaying different channel-forming activities (Blachly-Dyson *et al.*, 1993, 1997; Heins *et al.*, 1994;

Elkeles *et al.*, 1997; Sampson *et al.*, 1997; Xu *et al.*, 1999). Although the VDAC channel is formed from a single VDAC (porin) protein (Benz, 1994; Colombini *et al.*, 1996; Mannella, 1997; Blachly-Dyson and Forte, 2001), a number of studies have shown that the channel is the coordination point for large protein complexes such as the contact sites or the permeability transition pore (Brdiczka, 1991; Blachly-Dyson and Forte, 2001; Crompton *et al.*, 2002). In contrast, the assembly of the functional TOM complex channel requires the cooperation of several subunits (Ahting *et al.*, 1999; van Wilpe *et al.*, 1999; Paschen and Neupert, 2001; Pfanner and Chacinska, 2002; Rapaport, 2002).

Most of the knowledge concerning the TOM complex has been obtained by studying *Saccharomyces cerevisiae* and *Neurospora crassa* mitochondria. There are also some data concerning TOM complexes from mammals and plants pointing to their similarities in the overall structure to *S. cerevisiae* and *N. crassa* complexes (Mori and Terada, 1998; Braun and Schmitz, 1999; Suzuki *et al.*, 2000; Paschen and Neupert, 2001; Pfanner and Chacinska, 2002; Rapaport, 2002). The TOM complex contains surface receptors for the specific recognition of imported proteins and a general import/insertion pore (GIP), termed

[1] Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Fredry 10, 61-701 Poznan, Poland.

[2] To whom correspondence should be addressed; e-mail: kmita@ main.amu.edu.pl.

here the TOM complex channel, which mediates translocation of all nuclear-encoded mitochondrial preproteins into or across the outer membrane. In *S. cerevisiae* mitochondria, two surface receptors have been identified, namely Tom70 and Tom20, whereas the GIP consists of five proteins: Tom40, Tom22, Tom7, Tom6, and Tom5. Tom40, a major component of the TOM complex, forms the preprotein-conducting channel (Meisinger *et al.*, 1999; Hill *et al.*, 1998; Kunkele *et al.*, 1998a,b; Ahting *et al.*, 1999, 2001), while the central receptor Tom22 together with Tom7, Tom6, and Tom5 are associated tightly with the channel and are important for its activity (Rapaport *et al.*, 1998; Ahting *et al.*, 1999; van Wilpe *et al.*, 1999; Paschen and Neupert, 2001; Pfanner and Chacinska, 2002; Rapaport, 2002). Moreover, it has been shown recently that surface receptors may be crucial to the structural organization of the TOM complex, and thus also to the activity of the TOM complex channel (Model *et al.*, 2002). Since the electrophysiological characteristic of the TOM complex channel resembles that of the previously described PSC channel (peptide-sensitive channel), these channels are thought to be identical (Fevre *et al.*, 1990; Künkele *et al.*, 1998a,b).

The outer membrane of *S. cerevisiae* mitochondria besides the TOM complex channel contains only one isoform of the VDAC channel. It is formed by VDAC1 (or porin 1) encoded by the *POR1* gene (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998) and its properties are highly conserved among other species. It has been reported that in the absence of VDAC1, permeability of the outer membrane of *S. cerevisiae* mitochondria to external NADH is reduced 20-fold but still enables the access of the substrate to the intermembrane space (Lee *et al.*, 1998). Since the second VDAC protein present in *S. cerevisiae* mitochondria (called VDAC2 or porin2 and encoded by *POR2* gene) does not form a channel and plays a minimal role in external NADH permeability (Lee *et al.*, 1998), the finding is pertinent to the ability of the TOM complex channel to participate in metabolite transport across the mitochondrial outer membrane. We have shown recently that genetic elimination of the VDAC channel or its insufficient permeability results in the contribution of the TOM complex channel to external NADH transport into the intermembrane space of *S. cerevisiae* mitochondria (Kmita and Budzińska, 2000; Antos *et al.*, 2001a,b). Thus, the TOM complex channel can attenuate limitations in metabolite permeation through the mitochondrial outer membrane. In the case of mitochondria devoid of the VDAC channel (Δ*por1* mitochondria) the additional function of the TOM complex channel seems to be facilitated by the upregulation of the TOM complex components (Kmita and Budzińska, 2000; Antos *et al.*, 2001a). The upregulation

leading to higher levels of TOM complexes probably allows efficient transport of metabolites as well as proteins into Δ*por1* mitochondria. This, in turn, enables growth of Δ*por1* cells on nonfermentable carbon source, which proceeds with only a small delay when compared to wild type cells (e.g. Michejda *et al.*, 1990; Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998). Therefore, it is interesting to study processes underlying the upregulation of the TOM complex components in Δ*por1* mitochondria of *S. cerevisiae*.

Here, we report that Δ*por1* cells of *S. cerevisiae* contain distinctly increased levels of Tom40mRNA and Tom70mRNA, which can be regarded as the starting-point for the upregulation of the respective proteins. However, inhibitors of transcription and translation affect the levels of the mRNAs and the encoded proteins to different degrees. Thus, one could conclude that the absence of the VDAC channel triggers, probably indirectly, a distinct increase in the levels of mRNAs encoding the TOM complex components, although the mRNAs can be synthesized with different intensity and can display different stability. Consequently, the VDAC channel seems to be involved in the expression of components of the TOM complex.

MATERIALS AND METHODS

Yeast Strain

Two *S. cerevisiae* strains were used: the parental *POR1* strain M3 (*MATa, lys2 his4 trp1 ade2 leu2 ura3*) and M22-2 (Δ*por1*) containing a deletion of most of the POR1 gene (Blachly-Dyson *et al.*, 1997; Lee *et al.*, 1998).

Isolation of Mitochondria

Yeast cells were grown at 28°C in YPG medium (1% yeast extract, 2% peptone, 3% glycerol) at pH 5.5 and mitochondria were isolated according to the published procedures (Daum *et al.*, 1982). When inhibition of transcription or translation was performed, spheroplasts were incubated in a buffer containing 30 mM phosphate buffer pH 5.7, 250 mM sucrose, 3% glycerol at 28°C for 1 h in the presence of α-amanitin (10 μg/mL) or cycloheximide (7.5 μg/mL), respectively. The estimation of the integrity of the outer mitochondrial membrane was based on the permeability of the membrane to exogenous cytochrome *c* (Douce *et al.*, 1984) or on immunodecoration of Western blots with antisera against yeast marker proteins of different mitochondrial compartments. The calculated mean value of the degree of the outer membrane intactness was 96 and 94% for wild type and Δ*por1* mitochondria, respectively.

Isolation of mRNA and Dot Blot Hybridization

Spheroplasts preincubated in the absence or in the presence of α-amanitin (as described above) were used to isolate total mRNA with oligodT columns (Sigma). After precipitation, mRNA was denatured for 15 min at $55°$C in $0.5 \times$ MOPS buffer (for Northern blotting), in the presence of 2.2 M formaldehyde and 50% formamide and then transferred to Hybond-N+ membranes (Amersham) in the amount of 100 ng for one spot. The membranes were then baked for 2 h at $80°$C and used for hybridization with DNA probes (about 60–100 ng for one hybridization) labelled with psoralen-biotin, Ambion. Hybridization products were visualized by fluorography following detection with streptavidin-AP and CDPStar and quantified by ScanPack 3.0.

Synthesis of DNA Probes for Dot Blot Hybridization

Fragments of *TOM40*, *TOM70*, and *CC$_1$HL* genes, 590, 411, and 532 bp, respectively, were amplified by the PCR method in the presence of genomic DNA (isolated from wild type cells) as a template and primers designed using DNAStar and NCBI database. The amplified fragments were cut from agarose gel, purified (Qiagen columns) and labelled with psoralen-biotin (Ambion). It was also proved that each of the obtained probes is only complementary to a sequence of the respective gene and each of the genes is present in a single copy (yeast database of Stanford Genomic Resources).

Other Methods

Protein concentrations were measured by the methods of Bradford. Mitochondrial proteins separated by SDS-PAGE (Lämmli, 1970) were visualized by the ECL method following immunodecoration with anti-yeast antisera and quantified by ScanPack 3.0. Respiration of mitochondria was monitored at $25°$C with Rank oxygen electrode at an incubation volume of 0.5 mL. Changes of the inner membrane potential ($\Delta\Psi$) were monitored with tetraphenylophosphonium (TPP$^+$)-specific electrode (Kamo *et al.*, 1979).

RESULTS AND DISCUSSION

We have shown recently that in *S. cerevisiae* mitochondria depleted of the VDAC channel ($\Delta por1$ mitochondria) the TOM complex proteins are upregulated, which probably enables the complex involvement in metabolite transport across the outer membrane. The upregulation was shown for two chosen subunits of the complex, namely Tom40 (a channel forming subunit) and Tom70 (forming a surface receptor) (Kmita and Budziśka, 2000; Antos *et al.*, 2001a). The increased amounts of proteins in cells may result from their increased expression, i.e., transcription and translation as well as from enhanced "life-time" of products of these processes. Taking these rules into account, to discover processes underlying the upregulation of Tom40 and Tom70 we have decided to study the levels of *TOM40* and *TOM70* transcription and the respective mRNA translation in wild type and the VDAC channel-depleted ($\Delta por1$) cells.

Initially, we studied the levels of Tom40mRNA and Tom70mRNA in wild type and $\Delta por1$ cells by dot blot hybridization (as described in Materials and Methods section), a well-known method for detecting mRNA levels. Since cytochrome c_1 heme lyase (CC$_1$HL) was not observed to be upregulated in $\Delta por1$ mitochondria (Antos *et al.*, 2001a), CC$_1$HLmRNA was used as a control. As shown in Fig. 1(A) (α-amanitin) and Fig. 1(B), the amounts of Tom40mRNA and Tom70mRNA in $\Delta por1$ cells were distinctly higher than in wild type cells, approximately 1.9 and 2.3 times, respectively. The different levels of the increase in Tom40mRNA and Tom70mRNA amounts in $\Delta por1$ cells seems to correspond to the previously reported difference in the levels of Tom40 and Tom70 upregulation in $\Delta por1$ mitochondria, i.e., in the case of Tom70 the upregulation was stronger than in the case of Tom40 (Antos *et al.*, 2001a). The higher amounts of Tom40mRNA and Tom70mRNA in $\Delta por1$ cells could suggest that the absence of the VDAC channel triggers an increased synthesis or stabilization of mRNAs encoding the TOM complex components. However, surprisingly, the amount of CC$_1$HLmRNA in $\Delta por1$ cells was also increased in comparison with wild type cells, although the increase (approximately 1.3 times) was much less pronounced than in the case of Tom40mRNA and Tom70mRNA. The increased amount of CC$_1$HLmRNA in $\Delta por1$ cells which does not result in the upregulation of CC$_1$HL in $\Delta por1$ mitochondria might suggest that the mitochondria display a higher turnover of some proteins in comparison with wild type mitochondria. On the other hand, it can not be excluded that the slightly increased amount of CC$_1$HLmRNA results in a slight upregulation of CC$_1$HL, which is not detected by the applied method (Western blot). Nevertheless, the distinctly increased amounts of Tom40mRNA and Tom70mRNA in $\Delta por1$ cells are in agreement with the upregulation of the respective proteins in $\Delta por1$ mitochondria. Thus, the upregulation seems to start at the level of transcription.
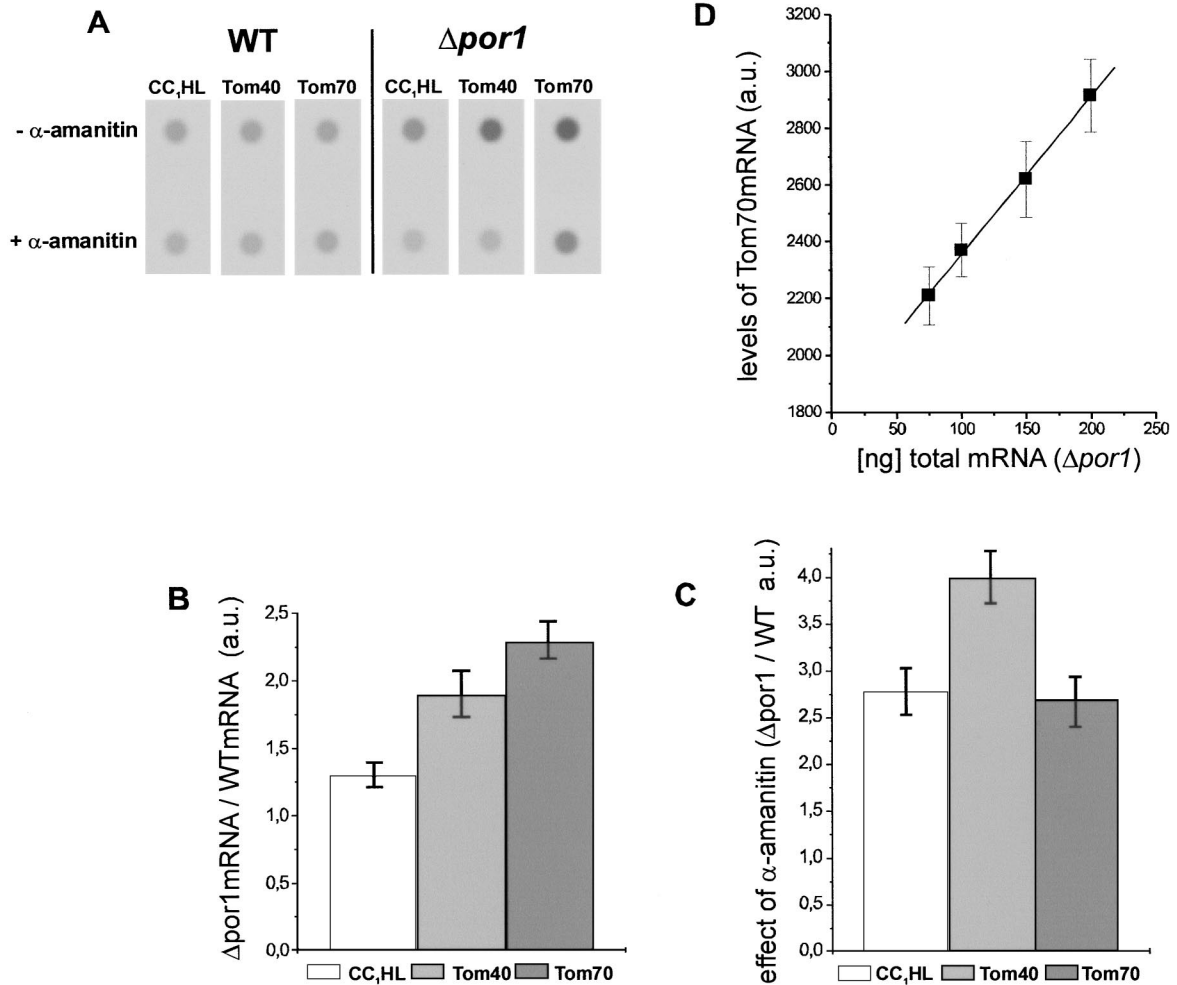
Fig. 1. The levels of Tom40mRNA, Tom70mRNA, and CC_1HLmRNA in wild type and $\Delta por1$ cells incubated in the presence and in the absence of α-amanitin. (A) Typical results of dot blot hybridization of total mRNA (100 ng) isolated from wild type or $\Delta por1$ spheroplasts, incubated in the presence or in the absence of α-amanitin, with the respective DNA probes. (B) Differences in the levels of the studied mRNAs between wild type and $\Delta por1$ spheroplasts in the absence of α-amanitin. (C) Differences in the effect of α-amanitin on the levels of the studied mRNAs between wild type and $\Delta por1$ cells. Data shown in (B) and (C) are mean values $\pm$ SEM of five independent experiments. (D) An example of test performed to ensure that the data shown in (A) were collected in the linear range of the applied methods. Results shown in (D) are mean values $\pm$ SEM of three independent experiments performed for total mRNA isolated from $\Delta por1$ spheroplasts. Quantitative analysis of dot blot hybridization results was performed by ScanPack3.0.

Further, the absence of the VDAC channel might play the role of a signal responsible for enhanced transcription or mRNA stabilization of nuclear-encoded mitochondrial proteins, depending on the function of the proteins.

To distinguish between processes contributing to an increased amount of mRNA, we determined the effect of α-amanitin, a well-known inhibitor of RNA polymerase II, on the levels of the studied mRNAs in wild type and $\Delta por1$ cells. As shown in Fig. 1(A), incubation with α-amanitin led to a decrease in the levels of the studied

mRNAs, which was much more clearly pronounced in $\Delta por1$ cells. This points to the higher levels of transcription of the studied mitochondrial protein genes in $\Delta por1$ cells. On the other hand, the calculated values of the ratios of the effect of α-amanitin on mRNA levels in $\Delta por1$ to wild type cells (Fig. 1(C)) indicated that in $\Delta por1$ cells the inhibitor affected Tom70mRNA and CC_1HLmRNA synthesis at comparable levels. Moreover, the effect was weaker than in the case of Tom40mRNA. Therefore, it might be concluded that in $\Delta por1$ cells, Tom40mRNA is synthesized more intensively than Tom70mRNA and the

synthesis of the latter proceeds probably at the same level as CC$_1$HLmRNA.

To check whether Tom40mRNA and Tom70mRNA may be synthesized with different intensity in $\Delta por1$ cells, we studied effects of α-amanitin and cycloheximide, a known inhibitor of translation, on the levels of the respective proteins in wild type and $\Delta por1$ mitochondria. As expected, the effects of α-amanitin and cycloheximide on Tom40 and Tom70 levels were more clearly pronounced in the case of $\Delta por1$ mitochondria (Fig. 2(A) and (B)), which confirms the enhanced expression of the studied proteins. Furthermore, when compared with wild type mitochondria, cycioheximide decreased the level of Tom70 in $\Delta por1$ mitochondria more distinctly than α-amanitin
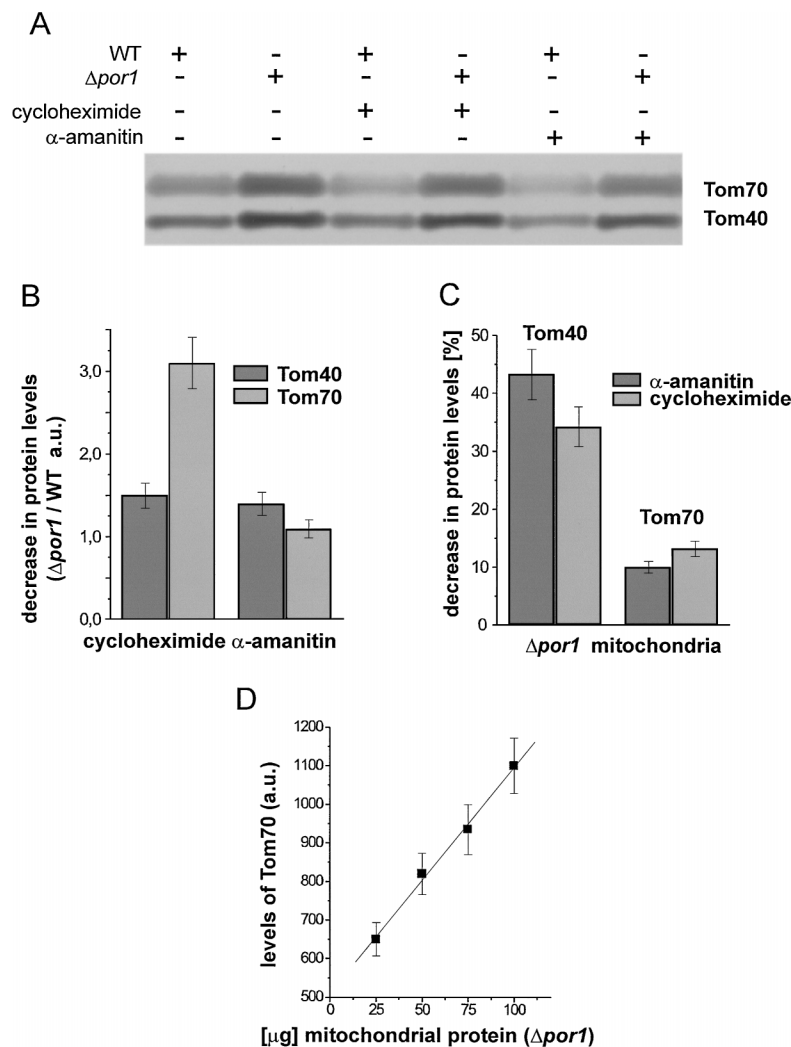
Fig. 2. The effect of α-amanitin and cycloheximide on the levels of Tom40 and Tom70 in wild type and $\Delta por1$ mitochondria. (A) Typical results of Western blot of the studied mitochondria (50 μg), isolated from spheroplasts incubated in the presence and in the absence of α-amanitin or cycloheximide, with anti-yeast mitochondrial protein antisera. (B) Differences in the effects of α-amanitin and cycloheximide on the levels of Tom40 and Tom70 between wild type and $\Delta por1$ mitochondria. (C) Quantitative analysis of the Western blot results obtained for $\Delta por1$ mitochondria. Data shown in (B) and (C) are mean values $\pm$ SEM of six independent experiments. (D) An example of test performed to ensure that the data shown in (A) were collected in the linear range of the applied methods. Results shown in (D) are mean values $\pm$ SEM of three independent experiments performed for $\Delta por1$ mitochondria. Quantitative analysis of Western blot results was performed by ScanPack3.0.

did. In the case of Tom40, the calculated ratios of the effects of both inhibitors on the protein levels in $\Delta por1$ to wild type mitochondria were nearly the same. The data are in agreement with the results shown in Fig. 2(C). The amount of Tom70 in $\Delta por1$ mitochondria decreased more strongly due to incubation with cycloheximide, while the level of Tom40 was decreased more powerfully in the presence of α-amanitin. Thus, the upregulation of Tom70 in $\Delta por1$ mitochondria might be explained by the increased translation of Tom70mRNA displaying increased stability, whereas the upregulation of Tom40 in the mitochondria results probably from the increased transcription of Tom40mRNA and its consecutive increased translation.

The results obtained indicate that in the case of *S. cerevisiae* cells, depletion of the VDAC channel causes an increase in the levels of mRNAs encoding components of the TOM complex which results in the upregulation of the respective proteins. This, in turn, probably allows the outer membrane permeability to adopt to the cellular needs. It could be speculated that $\Delta por1$ cells undergo simply a selective pressure to upregulate the VDAC channel like function for the TOM complex channel and this is reflected in the levels of the TOM complex components. However, the upregulation results from changes in the respective gene expression. Thus, in the absence of the VDAC channel, a signalling pathway leading to the upregulation has to be triggered. On the other hand, it can not be excluded that the presence of the VDAC channel might protect cells from the upregulation. Therefore, the VDAC channel might play an important role in the communication between mitochondria and nucleus. This function of the VDAC channel could be indirect through proteins associated with the channel, such as the one reported recently for a protein termed TspO, located in the outer membrane of a facultative photoheterotrophic bacterium *Rhodobacter sphaeroides* (Zeng and Kaplan, 2001). The TspO is involved in controlling transcription of a number of genes which encode enzymes involved in photopigment biosynthesis, although the effect of the protein appears to be through the repressor/antirepressor system, PpsR/AppA. Surprisingly, the TpsO displays a high degree of homology to the mitochondrial benzodiazepine receptor present in mammalian mitochondria within the protein complexes involving the VDAC channel.

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